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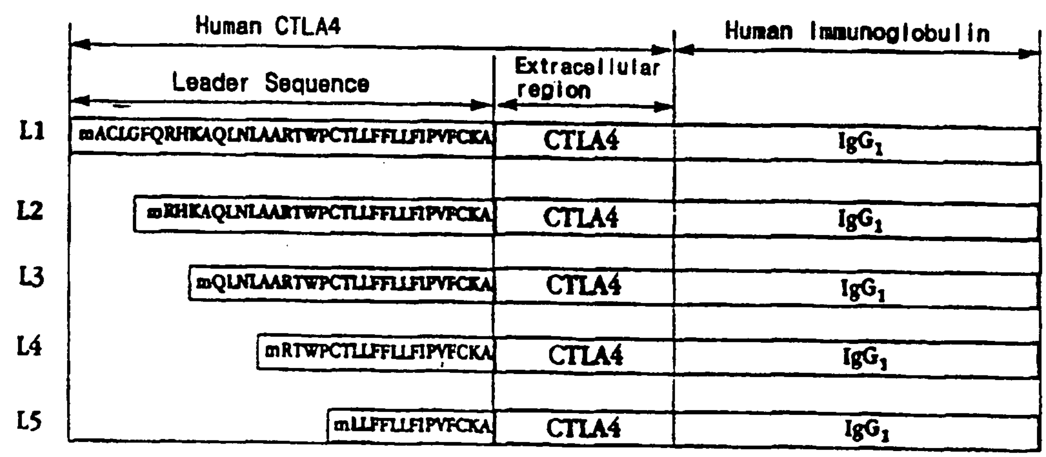

(57) Abstract

The present invention relates to a CTLA4–Ig fusion protein, in which an extracellular region of the CTLA4 is connected to CH_2, CH_3, and CH_4 of IgM or to hinge, CH_2 and CH_3 of IgG1 Cys_{308}, and six monomers of which are polymerized to be a hexameric structure. According to the present invention, it is provided a CTLA4–Ig fusion protein having a decreased dosage and high titer.

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A CTLA-4 Ig FUSION PROTEIN HAVING HIGH TITER

Technical Field

The present invention relates to a CTLA4-Ig fusion protein having high titer, and more particularly, to a fusion protein connecting an extracellular region of CTLA4 and C μ of IgM or C γ 1 region of IgG.

Background Art

In organ transplant, fatal to a patient is the rejection by immunoreaction which occurs by discriminating self and non-self.

In the rejection of the organ transplant, T-cell plays an important role. The reaction of T-cell starts with two kinds of signals, an antigen-sensitive stimulatory and a costimulatory signals. A large number of ligand/receptor bonds including ICAM-1/LFA-1, B7/CD28 and CTLA4 and LFA-3/CD2 participate in the costimulatoion. Especially, CD28 plays an important role in the reaction of the T-cell, making stable mRNA of a T-cell cytokinin by binding to the B7.1 and B7.2(June, C. H. *et al.*, *Mol. Cell Biol.*, 7, 4472, 1987/Lindstent, *et al.*, *Science*, **244**, 339, 1989), and increasing the productivity of interleukin-2(IL-2), interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), lymphotoxin, granulocyte macrophage-colony stimulating factor(GM-CSF), and interleukin-3(IL-3).

Thus if the costimulation by the CD28 is blocked by inhibiting binding of the CD28 and the B7.1 and B7.2, the rejection of the organ transplant can be suppressed.

CTLA4 has 67% homology with the CD28, binding to the B7(B7.1 and B7.2) of an antigen presenting cell(APC) like CD28. Linsley *et al.* reported that a monomeric CTLA4-Ig fusion protein was prepared by fusing the CTLA4 and an IgG, and that the protein has the immunosuppression effect (Linsley, P. S. *et al.*, *J. Exp. Med.* **174**, 561, 1991). Yamada *et al.* recently reported that they manufactured a

pentameric CTLA4-IgM fusion protein and that the protein extended lives of patients after the organ transplant (Yamada, A. *et al., Microbio. Immunol.*, **40**, 513~518, 1996)

However, the CTLA4-Ig fusion protein, since its too much dosage of 600 mg per once for a 60 kg adult and high manufacturing cost, is hardly commercially viable.

Disclosure of the Invention

According to one aspect of the present invention, there is provided a CTLA4-Ig fusion protein in which an extracellular region is connected with CH_2, CH_3, and CH_4 region of IgM, or with a hinge, CH_2, and CH_3 of IgG1 Cys_{308}(IgG1 having Cys_{308}), and which has a hexameric structure.

The hexameric structure of the CTLA4-Ig fusion protein is caused by forming multimer between adjoining IgMs or between IgG1 Cys_{308}s forced by disulfide bonds of cysteins. To put it concretely, Cys_{414} and Cys_{567} of the IgM make a disulfide bond and, in case of IgG1 Cys_{308}s of IgG1s make a disulfide bond. The IgG1 Cys_{308} of the present invention is the one that Leu_{308} of the IgG1 CH_2 region, the correspondent site of Cys_{414} of IgM, is converted to cystein in order to form polymeric IgG1 like IgM.

According to another aspect of the present invention, there is provided DNA base sequence coding amino acid sequence correspondent to the CTLA4-Ig fusion protein.

According to still another aspect of the present invention, there is provided the expression vectors pHIGH3neo and pHIGHgpt manufactured by inserting to vectors of pSV2neo and pSV2gpt an enhancer, a promoter, CTLA4 leader sequence of which N-terminal is cut, and DNA sequence coding amino acid sequence correspondent to the CTLA4-Ig fusion protein. The CTLA4 leader sequence of which N-terminal is cut makes the CTLA-Ig fusion protein secreted to the outside of cell.

According to still another aspect to the present invention, there is provided a transformed body manufactured by inserting to a mouse SP2/0-Ag14 cell the expression vectors pHIGH3neo and pHIGH3gpt which is manufactured by inserting to the vectors pSV2neo and pSV2gpt an enhancer, a promoter, CTAL4 leader sequence of which N-terminal is cut, and the DNA sequence coding amino acid sequence correspondent to the CTLA4-Ig fusion protein.

According to still another aspect of the present invention, there is provided an immunosuppressant containing the CTLA4-Ig fusion protein. The CTLA4-Ig fusion protein of the present invention, a soluble protein, binds to the B7 of the antigen presenting cell to inhibit binding of the CTLA4 and the CD28 of T-cell at the B7, to block costimulatory signal needed for the activation of T-cell and, in the result, the immunoreaction is suppressed.

By the features of the present invention, the titer of the CTLA4-Ig fusion protein according to the present invention is 32~356 times of an existing CTLA4-Ig fusion protein. The dosage of the CTLA4-Ig fusion protein according to the present invention is 2~13 mg per once for a 60 kg adult, and it's effective titer is 45~260 times of the existing CTLA4-Ig fusion protein's.

Brief Description of the Drawings

The above objects, and other features and advantages of the present invention will become more apparent after a reading of the following detailed description taken in conjunction with the drawings, in witch:

Fig.1 is a structure of a CTLA4 gene cloned by a reverse transcription-polymerase chain reaction(RT-PCR) of example 1.

Fig.2 is a expression ratio of a fusion protein of example 2.

Fig. 3a, 3b are base sequences of a CTLA4-IgM fusion gene of example 2 and an correspondent amino acid sequence thereof.

Fig. 4a, 4b are base sequence of a CTLA4-IgG1 Cys_{308} fusion gene of example 3 and a correspondent amino acid sequence thereof.

Fig. 5a, 5b are a manufacturing method for the expression vectors of pHIGH3neo and pHIGH3gpt of the CTLA4-IgM fusion gene and the CTLA4-IgG1 Cys_{308} fusion gene.

Fig. 6a, 6b are western blots of the CTLA4-IgM fusion protein and the CTLA4-IgG1 Cys_{308} fusion protein.

Fig. 7 is a structure of 600kD of the CTLA4-IgM fusion protein or the CTLA4-IgG Cys_{308} fusion protein.

Fig. 8 is a graph showing the immunosuppression effect of the CTLA4-IgM fusion protein and the CTLA4-IgG1 Cys_{308} fusion protein.

Best Mode for Carrying out the Invention

The present invention is further illustrated in the following example, which should not be taken to limit the scope of the invention.

Example 1: Cloning of human CTLA4, IgG1, and IgM genes

CTLA4, IgG1, and IgM genes were cloned respectively by the method of a reverse transcription-polymerase chain reaction(RT-PCR).

1. Cloning of the CTLA4 gene

A template used for the cloning of the CTLA4 gene by the reverse transcription-polymerase chain reaction was mononucleocyte mRNA of a healthy adult. The mRNA was separated as follows:

Blood taken from a healthy adult was density-gradient centrifuged using Ficoll-Hypaque to obtain monocyte cell layer. By adding RPMI-1640 medium containing 10% bovine fetus to the above monocytes $5X10^5$ monocytes/ml was made and here leukoagglutinin(Pharmacia Corp.) added to be 3.5 μg/ml. The mixture was incubated 36~48 hours under the condition of 5% CO_2, 37℃ in order to separate mRNA.

The polymerase used in the reverse transcription-polymerase chain reaction was pfu(Stratagene Corp.).

The primers used in the reverse transcription-polymerase chain

reaction are five forward primers(L1~5) and a reverseward primer, as follows;

Forward primers

L1 5'-ATG GCT TGC CTT GGA TTT CAG-3'

L2 5'-ATG CGG CAC AAG GCT CAG CTG AAC-3'

L3 5'-ATG CAG CTG AAC CTG GCT GCC AGG-3'

L4 5'-ATG AGG ACC TGG CCC TGC ACT CTC-3'

L5 5'-ATG CTC CTG TTT TTT CTT CTC TTC-3'

Reverseward primer

5'-CTC TGC AGA ATC TGG GCA CGG TTC AGG ATC-3'

It is invented for the L1 primer to be expressed as an original CTLA4 without cutting, for the L2 primer as a form that 6 amino acids of it were cut from N-terminal, 11 amino acids cut for the L3, 16 amino acids cut for the L4, and 22 amino acids cut for the L5 from N-terminal (Fig.1).

Inventing the forward primers to be expressed as cutting form of amino acids from N-terminal is for a part of leader sequence to be cut and expressed , and for the CTLA4 protein to be secreted to an extracellular region. And 5 primers were invented in order that the leader sequence is cut and expressed one by one for the determination of a leader sequence which makes the most CTLA4 proteins secreted to extracellular region.

CTLA4 gene obtained by the reverse transcription-polymerase reaction was cloned to pUC 18. The cloned CTLA4 gene has confirmed which base No.49 was converted from adenine to guanine, and base No.331 was converted from guanine to adenine. In the result, an amino acid No.17 of CTLA4 protein was converted from threonine to alanine, and an amino acid No.111 of CTLA4 protein was converted from alanine to threonine.

2. Cloning of IgG1 gene

The cloning method was same with the method of the above 1 of the example 1 except template and primer. The template used here was mRNA of B-cell at peripheral blood lymph node obtained from a recovering ill-defined fever patient. The primer was invented in order to clone a counterbalancing of IgG1 as follows;

Forward primer

5'-A TCT GCA GAG CCC AAA TCT TGT GAC-3'

Reverseward primer

5'-TT CTC GAG TCA TTT ACC CGG AGA CAG GGA-3'

3. Cloning of IgM gene

Same with the method of the above 2 of the example 1 except primer. The primer was invented in order to clone a counterbalancing of the IgM as follows;

Forward primer

5'-GAC TGC AGA GCT GCC TCC CAA AGT G-3'

Reverseward primer

5'-GTA GCA GGT GCC AGC TGT GTC TGA-3'

Example 2: Determination of the optimum leader sequence for extracellular secretion

The five CTLA4 genes obtained by serial deletion of N-terminal amino acids were fused with IgG1 respectively, inserted to a vector pHIGH3, and transfected to a mouse bone marrow SP2/0-Ag14 cell(ATCC#: CRL 1581) to be expressed. And after an incubation for 48 hours, the expression ratio was analyzed by a cell circulation assay.

The result of the above analysis shows that in case of L1 primer 4.9% of the fusion protein, 3.1% for L2 primer, 0% for L3 primer, 7.8% for L4 primer and 6% for L5 primer are expressed (Fig.2). It confirms that the leader sequence deleted of 16 amino acids from N-terminal, obtained by using L-4 primer, makes the most fusion proteins secreted

most to an extracellular region.

Example 3: Manufacturing of IgG1 Cys_{308}

IgG1 Cys_{308} was manufactured by converting Leu_{308} of IgG1 to cysteine using a polymerase chain reaction. The primers used in the polymerase chain reaction are as follows;

Forward primer
5'-A TCT GCA GAG CCC AAA TCT TGT GAC-3'
Reverseward primer
5'-TT CTC GAG TCA TTT ACC CGG AGA CAG GGA-3'
Converting primer
5'-CCA GTC CTG GTG ACA GAC GGT GAG GAC-3'

First, the primary polymerase chain reaction using the forward primer and reverseward primer was performed, and then using the product of the above reaction and reverseward primer, secondary polymerase chain reaction was performed. The amplified product of the secondary polymerase chain reaction was cloned in pUC18 vector.

Example 4: Construction of the expression vector of the CTLA4-Ig fusion gene

Genome DNA of SP2/0-Ag14 cell was extracted, cut with restriction enzymes of BamH I and Hind III, transferred to a nitrocellulose membrane, and performed Southern blot with 5'-ATT TGC ATA TTT GCA TAT TTG CAT-3' fragment and 5'-CTC ATG ACT CAT GAC TCA-3' fragment marked with isotope to clone 5.3kb promoter.

On the other hand genome DNA of SP2/0-Ag14 cell was cut by restriction enzymes of EcoR I and BamH I and performed the southern blot with 5'-TGA ATT GAG CAA TGT TGA ATT GAG CAA TGT-3' fragment and 5'-TAT TTG GGG AAG GGT ATT TGG GGA AGG-3' fragment marked with isotope to clone 1kb enhancer.

An enhancer-promoter-CTLA4-Ig fusion gene was cloned to pUC 18 by fusing the 1kb enhancer and 5.3 kb promoter in pUC 19, and inserting the fused product to the site of Sal I, the front part of CTLA4-Ig fusion gene cloned in pUC 18(CTLA4-IgM fusion gene of the example 2 and CTLA4-IgG1 Cys_{308} fusion gene of the example 3, Fig.3a,3b and Fig.4a, 4b). By cutting only enhancer-promoter-CTLA4-Ig fusion gene by treating EcoR I and Hind III to the above clone and then by inserting the clone to pSV2neo and pSV2gpt, the expression vectors of pHIGH3neo and pHIGH3gpt was constructed (Fig. 5a, 5b).

Example 5: Expression of CTLA4-Ig fusion gene and purification of CTLA4-Ig fusion protein

SP2/0-Ag14 cell of mouse was incubated in 10% FCS-DMEM medium, and diluted to $5X10^6$ cells/mℓ by adding PBS. The above suspension 0.2mℓ was put to cuvette(BioRad Corp.) for electroporation and the purified expression vector 15μg of the CTLA4-Ig fusion gene of example 4 was added. And then electroporation (BT×820) was performed under the condition of 480V, 99 μsec, 2cycle.

The above cells were incubated for 3 weeks in the FCS-DMEM medium containing 1500μg/mℓ of geneticin G418(Gibco Corp.). And then colonies were separated, collected, and incubated for amplifying. The CTLA4-Ig fusion gene expression was examined by the a cell circulation analyzer and enzyme linked immunosorbent assay(ELISA) method.

These cells were incubated in large quantities in FCS-medium and the CTLA4-Ig fusion protein was precipitated by ammonium sulfate addition. And then by an affinity chromatography using protein A, the CTLA4-Ig fusion protein was purified.

In order to fine out the biochemical properties of the CTLA4-Ig fusion protein, electrophoresis and western blot were performed(F

ig. 6a, 6b). The result shows that there are two kinds of the CTL A4-IgM fusion protein and six kinds of the CTLA4-IgG1 fusion protein. Among these, CTLA4-Ig fusion protein of 600kD was separated and purified. The CTLA4-Ig fusion protein of 600kD is 6 times as large as the existing CTL A4-Ig fusion protein(100kD), and is a hexamer which was six of CTL A4-Ig fusion protein polymerized (Fig.7).

Example 6: Immunosuppression effect of the CTLA4-Ig fusion protein

The existing CTLA4-Ig fusion protein is a comparative example 1, the pentameric CTLA4-Ig fusion protein is a comparative example 2, and the hexameric CTLA4-Ig fusion protein is an example. and the Immunosuppression effects of them were examined as follows;

From two healthy adults peripheral blood lymphocytes were separated, and on the cells of the one person 300 rad of ^{60}Co radiation was irradiated.

The cells of the two persons were spread into a 96-well plate with $2.5X10^4$ cells/ml, respectively. And after incubating for 88~96 hours under the condition of 37℃, 5% CO_2, added 0.5 μCi ^{3}H-thymidine(NEN Research product) per well and incubated 5 hours again.

The incubated cells were adsorbed to a glass filter by using titertek(Flow lab), put into a test tube, and after adding 5$\mu\ell$ of Scintillation cocktail a radioactivity was measured by using β-liquid scintillation counter. The all tests were performed three for every times under the same condition and an average of them was determined. The percent value gained by adding the fusion protein of the present invention was calculated on the basis of the radiation value(100%) gained without an addition. And when the value reaches to 50%, the value was defined as a line of 50% division suppression and the titer between fusion proteins was compared on the basis of the concentration of the adding

fusion protein.

As a result, the 50% division suppression concentration of the CTLA4-Ig fusion protein of this example is 0.009~0.022 μg/mℓ(the average is 0.016 μg /mℓ). This value is lower than 0.7~3.2 μg/mℓ(the average is 1.4 μg/mℓ) of the comparative example 1 and lower than 0.031~0.056 μg/mℓ (the average is 0.44 μg/mℓ) the comparative example 2 (Fig.8). CTLA4-Ig fusion protein of this example has high titer, 32~356 times (the average is 88 times) comparing to the existing CTLA4-Ig fusion protein of the comparative example 1 .

CLAIMS

1. A CTLA4-IgM fusion protein, wherein an extracellular region of a CTLA4 is connected with CH_2, CH_3, and CH_4 of IgM, and which has a hexameric structure by polymerization of 6 monomers thereof.

2. A DNA sequence of Fig.4a, 4b coding the amino acid sequence corresponding to the CTLA4-IgM fusion protein as claimed in Claim 1.

3. A expression vector pHIGH3neo which is constructed by connecting an enhancer, a promoter and a CTLA4 of which N-terminal is cut, and DNA sequence coding amino acid sequence correspondent to the CTLA4-IgM fusion protein of Claim 1, and then by inserting the DNA sequence into vector pSV2neo.

4. A expression vector pHIGH3neo as claimed in Claim 3, wherein 16 amino acids of a leader sequence are cut from N-terminal.

5. A transformed body manufactured by inserting to a mouse SP2/0-Ag14 the expression vector pHIGH3neo constructed by connecting an enhancer, a promoter and a CTLA4 of which N-terminal is cut, and DNA sequences coding amino acid sequence correspondent to the CTLA4-IgM fusion protein of Claim 1, and then by inserting them into vector pSV2neo.

6. An immunosuppression medicine containing the CTLA4-IgM fusion protein in Claim 1.

7. A CTLA4-IgG1 Cys_{308} fusion protein, wherein the extracellular region of CTLA4 is connected with a hinge, CH_2 and CH_3 of the IgG1 Cys_{308} (IgG1 having Cys_{308}) and which has a hexameric structure by polymerization of 6 fusion protein monomers thereof.

8. A DNA sequence of Fig.3a, 3b coding amino acid sequence correspondent to the CTLA4-IgG1 Cys_{308} fusion protein in Claim 7.

9. An expression vector pHIGH3neo which is constructed by connecting an enhancer, a promoter, and CTLA4 of which N-terminal is cut, and DNA sequence coding amino acid sequences correspondent to the CTLA4-IgG1 Cys_{308} fusion protein in Claim 7, and then by inserting

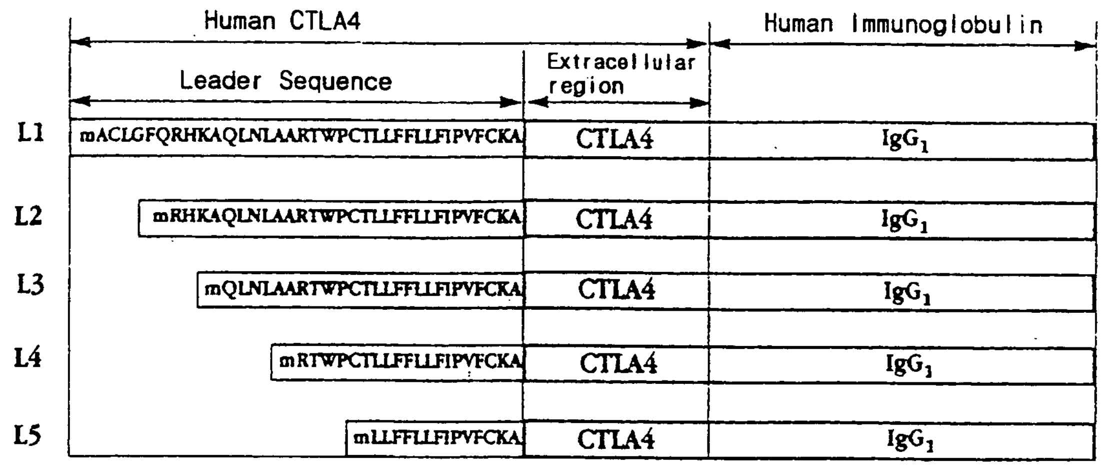
them into a vector pSV2neo.

10. An expression vector pHIGH3neo as claimed in Claim 9, which 16 amino acids of a leader sequence are cut from N-terminal.

11. A transformed body which is manufactured by inserting to a mouse SP2/0-Ag14 cell the expression vector pHIGH3neo constructed by connecting an enhancer, a promoter, and CTLA4 of which N-terminal is cut, and DNA sequence coding amino acid sequences correspondent to the CTLA4-IgG1 Cys_{308} fusion protein in Claim 7, and then by inserting them into a vector pSV2neo.

12. An immunosuppressant containing the CTLA4-IgG1 Cys_{308} fusion pr otein in Claim 7.

Fig. 1


Human CTLA4
Human Immunoglobulin
Leader Sequence
Extracellular region
L1
mACLGFQRHKAQLNLAARTWPCTLLFFLLFIPVFCKA
CTLA4
IgG_1
L2
mRHKAQLNLAARTWPCTLLFFLLFIPVFCKA
CTLA4
IgG_1
L3
mQLNLAARTWPCTLLFFLLFIPVFCKA
CTLA4
IgG_1
L4
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IgG_1
L5
mLLFFLLFIPVFCKA
CTLA4
IgG_1

Fig. 2

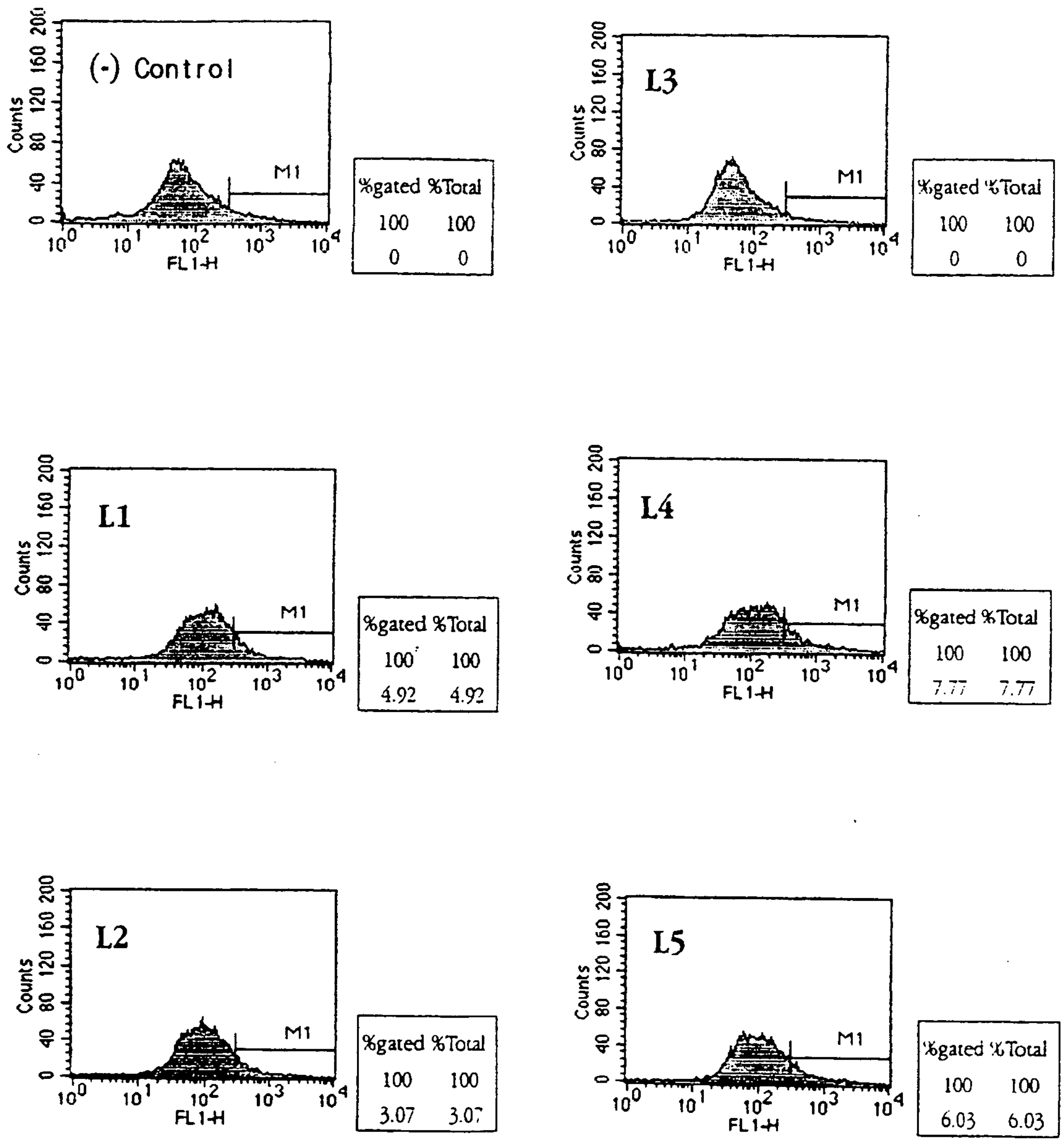

(-) Control
Counts
FL1-H
M1
%gated %Total
100 100
0 0
L3
%gated %Total
100 100
0 0
L1
%gated %Total
100 100
4.92 4.92
L4
%gated %Total
100 100
7.77 7.77
L2
%gated %Total
100 100
3.07 3.07
L5
%gated %Total
100 100
6.03 6.03

Fig. 3a

```
A   M   H   V   A   Q   P   A   V   V
GCA ATG CAC GTG GCC CAG CCT GCT GTG GTA
L   A   S   S   R   G   I   A   S   F
CTG GCC AGC AGC CGA GGC ATC GCC AGC TTT
V   C   E   Y   A   S   P   G   K   A
GTG TGT GAG TAT GCA TCT CCA GGC AAA GCC
T   E   V   R   V   T   V   L   R   Q
ACT GAG GTC CGG GTG ACA GTG CTT CGG CAG
A   D   S   Q   V   T   E   V   C   A
GCT GAC AGC CAG GTG ACT GAA GTC TGT GCG
A   T   Y   M   M   G   N   E   L   T
GCA ACC TAC ATG ATG GGG AAT GAG TTG ACC
F   L   D   D   S   I   C   T   G   T
TTC CTA GAT GAT TCC ATC TGC ACG GGC ACC
S   S   G   N   Q   V   N   L   T   I
TCC AGT GGA AAT CAA GTG AAC CTC ACT ATC
Q   G   L   R   A   M   D   T   G   L
CAA GGA CTG AGG GCC ATG GAC ACG GGA CTC
Y   I   C   K   V   E   L   M   Y   P
TAC ATC TGC AAG GTG GAG CTC ATG TAC CCA
P   P   Y   Y   L   G   I   G   N   G
CCG CCA TAC TAC CTG GGC ATA GGC AAC GGA
T   Q   I   Y   V   I   D   P   E   P
ACC CAG ATT TAT GTA ATT GAT CCA GAA CCG
C   P   D   S   A   E   P   K   S   C
TGC CCA GAT TCT GCA GAG CCC AAA TCT TGT
D   K   T   H   T   C   P   P   C   P
GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA
A   P   E   L   L   G   G   P   S   V
GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC
F   L   F   P   P   K   P   K   D   T
TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC
L   M   I   S   R   T   P   E   V   T
CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA
```

Extracellular region

CTLA-4

Hinge region

Fig. 3b

```
C   V   V   V   D   V   S   H   E   D
TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC
P   E   V   K   F   N   W   Y   V   D
CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC
G   V   E   V   H   N   A   K   T   K
GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG
P   R   E   E   Q   Y   N   S   T   Y
CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC
R   V   V   S   V   L   T   V   C   H
CGG GTG GTC AGC GTC CTC ACC GTC TGT CAC
Q   D   W   L   N   G   K   E   Y   K
CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG
C   K   V   S   N   K   A   L   P   A
TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC
P   I   E   K   T   I   S   K   A   K
CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA
G   Q   P   R   E   P   Q   V   Y   T
GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC
L   P   P   S   R   D   E   L   T   K
CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG
N   Q   V   S   L   T   C   L   V   K
AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA
G   F   Y   P   S   D   I   A   V   E
GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG
W   E   S   N   G   Q   P   E   N   N
TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC
Y   K   T   T   P   P   V   L   D   S
TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC
D   G   S   S   F   L   Y   S   K   L
GAC GGC TCC TCC TTC CTC TAC AGC AAG CTC
T   V   D   K   S   R   W   Q   Q   G
ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG
N   V   F   S   C   S   V   M   H   E
AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG
A   L   H   N   H   Y   T   Q   K   S
GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC
L   S   L   S   P   G   K   .
CTC TCC CTG TCT CCG GGT AAA TGA
```

CH_2 region

CH_3 region

IgG_1-Cys_{308}

Fig. 4a

```
A   M   H   V   A   Q   P   A   V   V
GCA ATG CAC GTG GCC CAG CCT GCT GTG GTA
L   A   S   S   R   G   I   A   S   F
CTG GCC AGC AGC CGA GGC ATC GCC AGC TTT
V   C   E   Y   A   S   P   G   K   A
GTG TGT GAG TAT GCA TCT CCA GGC AAA GCC
T   E   V   R   V   T   V   L   R   Q
ACT GAG GTC CGG GTG ACA GTG CTT CGG CAG
A   D   S   Q   V   T   E   V   C   A
GCT GAC AGC CAG GTG ACT GAA GTC TGT GCG
A   T   Y   M   M   G   N   E   L   T
GCA ACC TAC ATG ATG GGG AAT GAG TTG ACC
F   L   D   D   S   I   C   T   G   T
TTC CTA GAT GAT TCC ATC TGC ACG GGC ACC
S   S   G   N   Q   V   N   L   T   I
TCC AGT GGA AAT CAA GTG AAC CTC ACT ATC
Q   G   L   R   A   M   D   T   G   L
CAA GGA CTG AGG GCC ATG GAC ACG GGA CTC
Y   I   C   K   V   E   L   M   Y   P
TAC ATC TGC AAG GTG GAG CTC ATG TAC CCA
P   P   Y   Y   L   G   I   G   N   G
CCG CCA TAC TAC CTG GGC ATA GGC AAC GGA
T   Q   I   Y   V   I   D   P   E   P
ACC CAG ATT TAT GTA ATT GAT CCA GAA CCG
C   P   D   S   A   E   L   P   P   K
TGC CCA GAT TCT GCA GAG CTG CCT CCC AAA
V   S   V   F   V   P   P   R   D   G
GTG AGC GTC TTC GTC CCA CCC CGC GAC GGC
F   F   G   N   P   R   K   S   K   L
TTC TTC GGC AAC CCC CGC AAG TCC AAG CTC
I   C   Q   A   T   G   F   S   P   R
ATC TGC CAG GCC ACG GGT TTC AGT CCC CGG
Q   I   Q   V   S   W   L   R   E   G
CAG ATT CAG GTG TCC TGG CTG CGC GAG GGG
K   Q   V   G   S   G   V   T   T   D
AAG CAG GTG GGG TCT GGC GTC ACC ACG GAC
Q   V   Q   A   E   A   K   E   S   G
CAG GTG CAG GCT GAG GCC AAA GAG TCT GGG
P   T   T   Y   K   V   T   S   T   L
CCC ACG ACC TAC AAG GTG ACC AGC ACA CTG
T   I   K   E   S   D   W   L   G   Q
ACC ATC AAA GAG AGC GAC TGG CTC GGC CAG
S   M   F   T   C   R   V   D   H   R
AGC ATG TTC ACC TGC CGC GTG GAT CAC AGG
G   L   T   F   Q   Q   N   A   S   S
GGC CTG ACC TTC CAG CAG AAT GCG TCC TCC
M   C   V   P   D   Q   D   T   A   I
ATG TGT GTC CCC GAT CAA GAC ACA GCC ATC
R   V   F   A   I   P   P   S   F   A
CGG GTC TTC GCC ATC CCC CCA TCC TTT GCC
```

CTLA-4

Extracellular region

CH_2 region

Fig. 4b

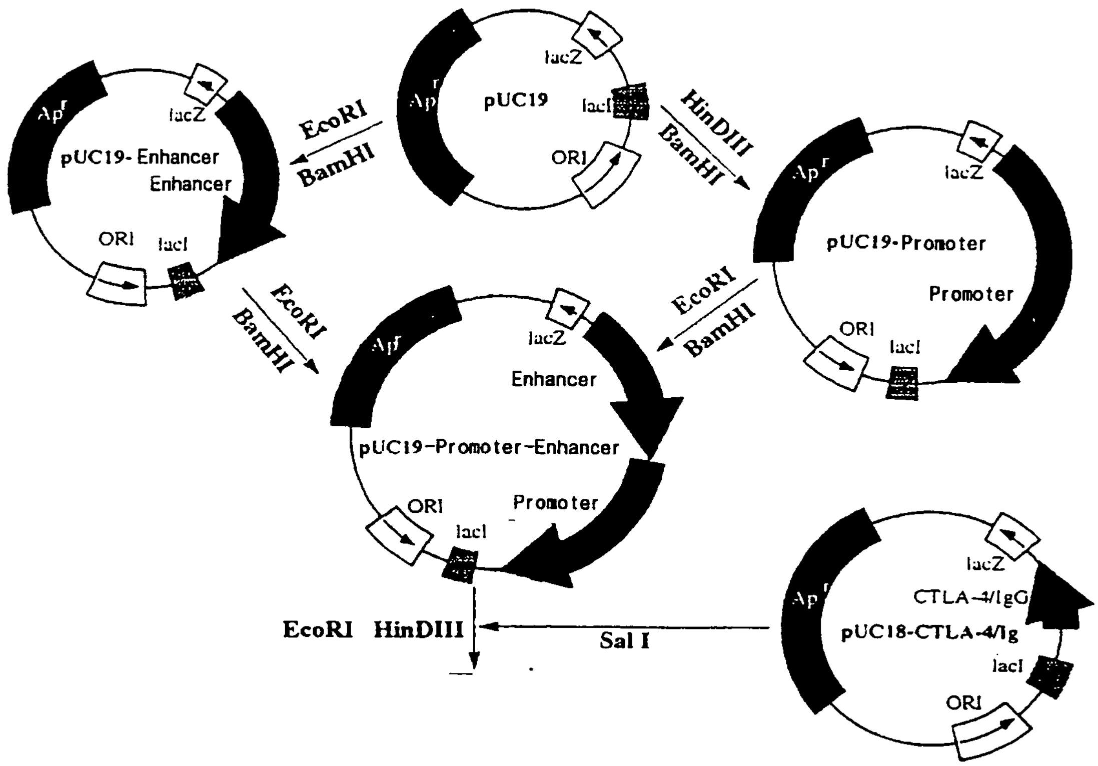
```
S   I   F   L   T   K   S   T   K   L
AGC ATC TTC CTC ACC AAG TCC ACC AAG TTG
T   C   L   V   T   D   L   T   T   Y
ACC TGC CTG GTC ACA GAC CTG ACC ACC TAT
D   S   V   T   I   S   W   T   R   Q
GAC AGC GTG ACC ATC TCC TGG ACC CGC CAG
N   G   E   A   V   K   T   H   T   N
AAT GGC GAA GCT GTG AAA ACC CAC ACC AAC
I   S   E   S   H   P   N   A   T   F
ATC TCC GAG AGC CAC CCC AAT GCC ACT TTC
S   A   V   G   E   A   S   I   C   E
AGC GCC GTG GGT GAG GCC AGC ATC TGC GAG
D   D   W   N   S   G   E   R   F   T
GAT GAC TGG AAT TCC GGG GAG AGG TTC ACG
C   T   V   T   H   T   D   L   P   S
TGC ACC GTG ACC CAC ACA GAC CTG CCC TCG
P   L   K   Q   T   I   S   R   P   K
CCA CTG AAG CAG ACC ATC TCC CGG CCC AAG
G   V   A   L   H   R   P   D   V   Y
GGG GTG GCC CTG CAC AGG CCC GAT GTC TAC
L   L   P   P   A   R   E   Q   L   N
TTG CTG CCA CCA GCC CGG GAG CAG CTG AAC
L   R   E   S   A   T   I   T   C   L
CTG CGG GAG TCG GCC ACC ATC ACG TGC CTG
V   T   G   F   S   P   A   D   V   F
GTG ACG GGC TTC TCT CCC GCG GAC GTC TTC
V   Q   W   M   Q   R   G   Q   P   L
GTG CAG TGG ATG CAG AGG GGG CAG CCC TTG
S   P   E   K   Y   V   T   S   A   P
TCC CCG GAG AAG TAT GTG ACC AGC GCC CCA
M   P   E   P   Q   A   P   G   R   Y
ATG CCT GAG CCC CAG GCC CCA GGC CGG TAC
F   A   H   S   I   L   T   V   S   E
TTC GCC CAC AGC ATC CTG ACC GTG TCC GAA
E   E   W   N   T   G   E   T   Y   T
GAG GAA TGG AAC ACG GGG GAG ACC TAC ACC
C   V   A   H   E   A   L   P   N   R
TGC GTG GCC CAT GAG GCC CTG CCC AAC AGG
V   T   E   R   T   V   D   K   S   T
GTC ACC GAG AGG ACC GTG GAC AAG TCC ACC
G   K   P   T   L   Y   N   V   S   L
GGT AAA CCC ACC CTG TAC AAC GTG TCC CTG
V   M   S   D   T   A   G   T   C   Y
GTC ATG TCC GAC ACA GCT GGC ACC TGC TAC
.
TGA
```

CH_3 region

CH_4 region

IgM

Fig. 5a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lacZ
pUC19- Enhancer
Enhancer
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ORI
HinDIII
BamHI
Ap^r
lacZ
pUC19-Promoter
Promoter
ORI
lacI
EcoRI
BamHI
EcoRI
BamHI
Ap^r
lacZ
Enhancer
pUC19-Promoter-Enhancer
ORI
Promoter
lacI
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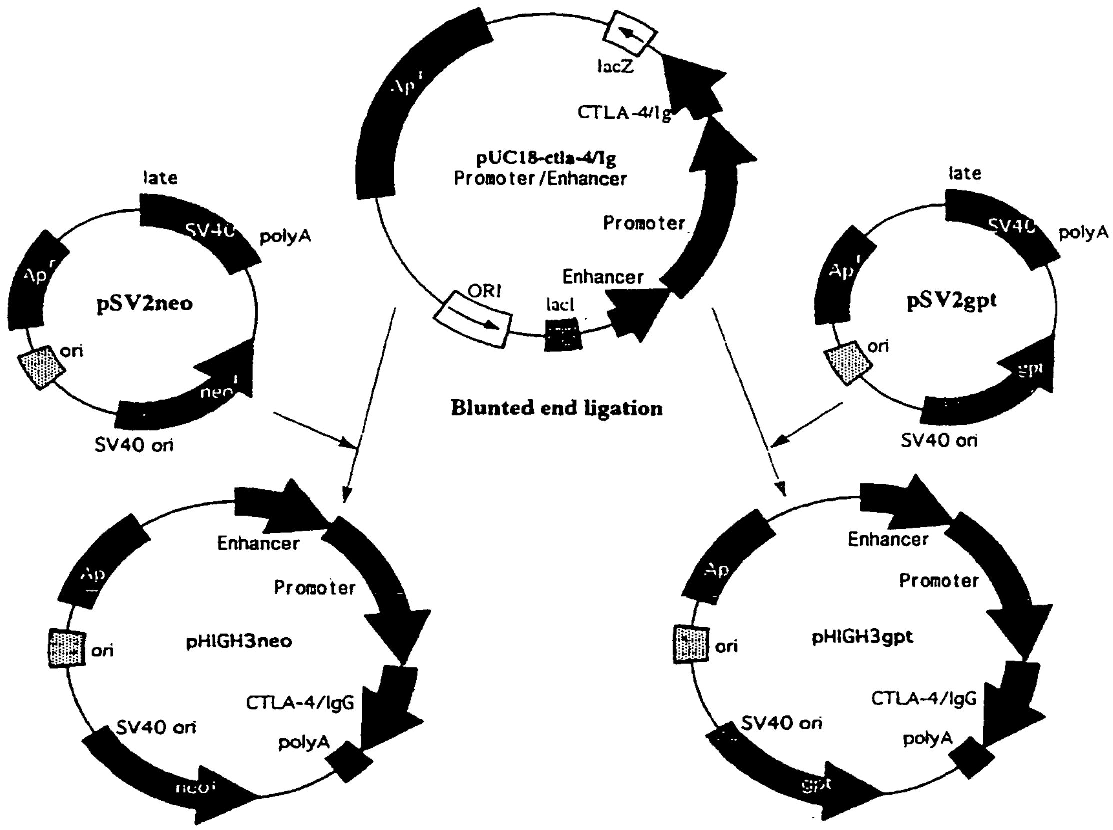
Sal I
Ap^r
lacZ
CTLA-4/IgG
pUC18-CTLA-4/Ig
lacI
ORI

Fig. 5b



pUC18-ctla-4/Ig
Promoter/Enhancer
lacZ
CTLA-4/Ig
Promoter
Enhancer
ORI
lacI
pSV2neo
late
SV40
polyA
ori
neo
SV40 ori
pSV2gpt
late
SV40
polyA
ori
gpt
SV40 ori
Blunted end ligation
pHIGH3neo
Enhancer
Promoter
ori
CTLA-4/IgG
SV40 ori
polyA
neo
pHIGH3gpt
Enhancer
Promoter
ori
CTLA-4/IgG
SV40 ori
polyA
gpt

Fig. 6a

A The properties of the CTLA4-IgG_1-Cys_{308} fusion protein

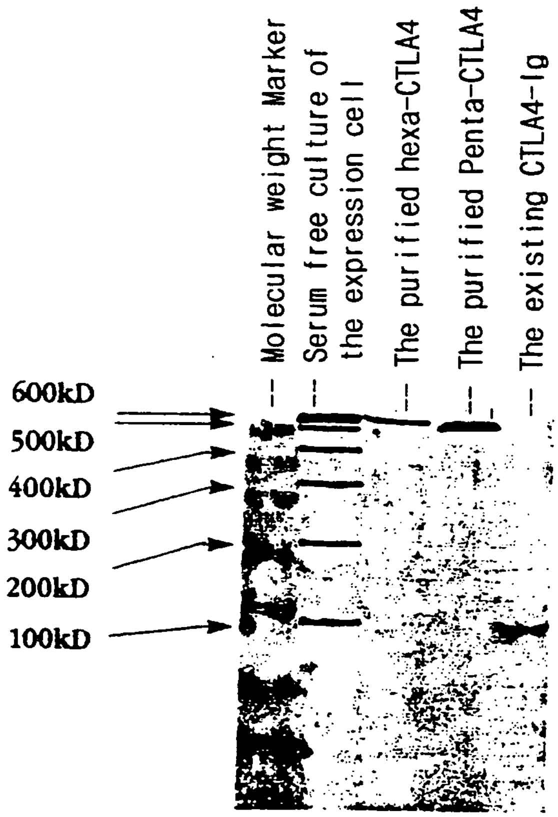


Fig. 6b

B The properties of the CTLA4-IgM fusion protein

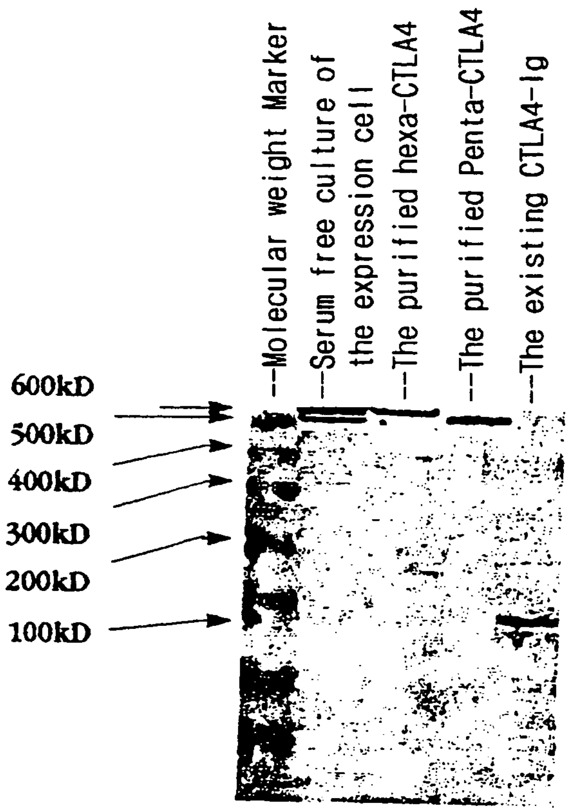


Fig. 7

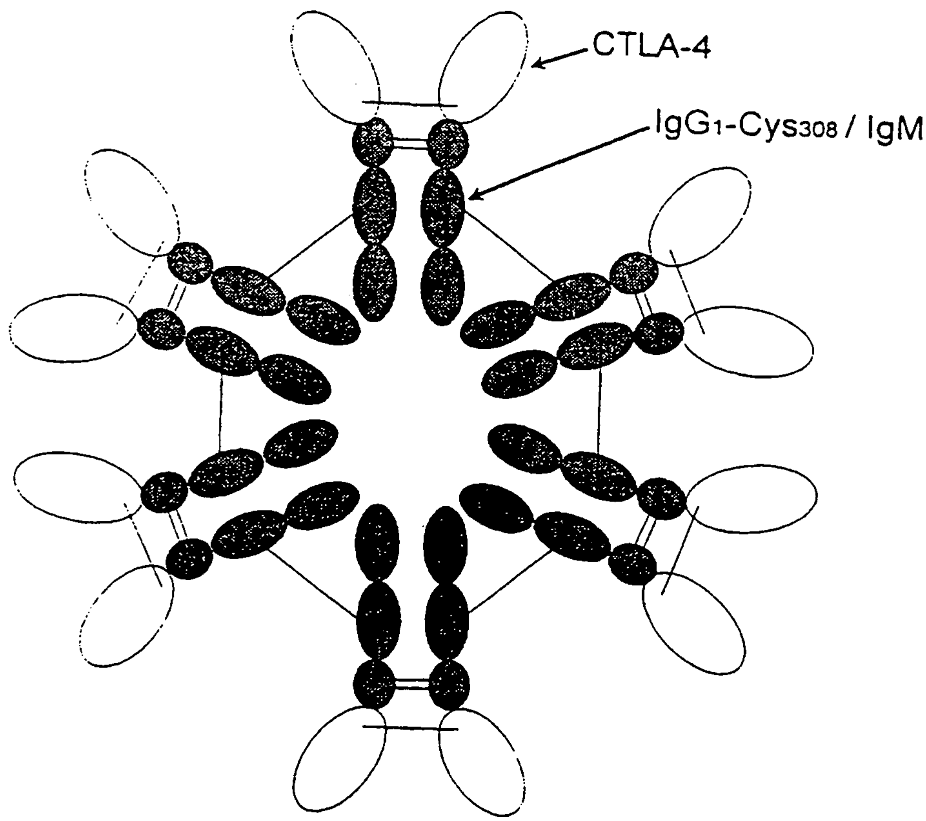
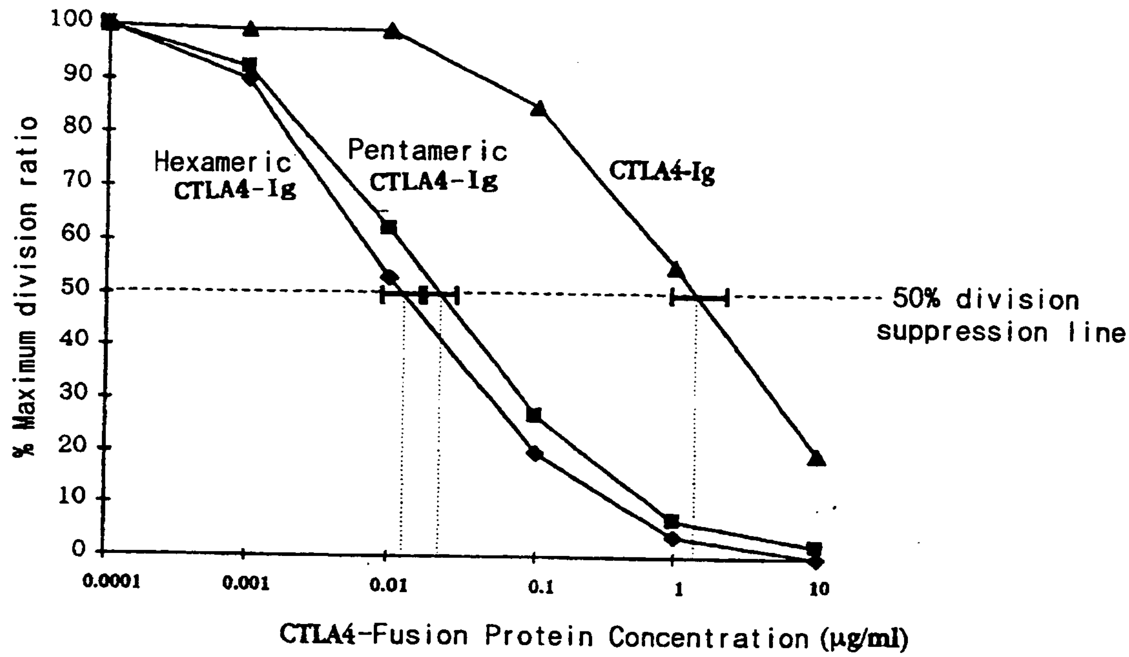


Fig. 8


100
90
80
70
60
50
40
30
20
10
0
% Maximum division ratio
Hexameric
CTLA4-Ig
Pentameric
CTLA4-Ig
CTLA4-Ig
50% division
suppression line
0.0001
0.001
0.01
0.1
1
10
CTLA4-Fusion Protein Concentration (μg/ml)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 98/00009

A. CLASSIFICATION OF SUBJECT MATTER

IPC^6: C 12 N 15/62; A 61 K 38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC^6: C 12 N 15/62; A 61 K 38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	US 5 434 131 A (LINSLEY et al.) 18 July 1995 (18.07.95), abstract; claims 5,6.	1,6

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 May 1998 (04.05.98)	15 May 1998 (15.05.98)

Name and mailing address of the ISA/ AT	Authorized officer
AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/535	Wolf Telephone No. 1/53424/436

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 98/00009

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